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# Targeted Transgenic Overexpression of Mitochondrial Thymidine Kinase (TK2) Alters Mitochondrial DNA (mtDNA) and Mitochondrial Polypeptide Abundance

## *Transgenic TK2, mtDNA, and Antiretrovirals*

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**Mitochondrial toxicity limits nucleoside reverse transcriptase inhibitors (NRTIs) for acquired immune deficiency syndrome. NRTI triphosphates, the active moieties, inhibit human immunodeficiency virus reverse transcriptase and eukaryotic mitochondrial DNA polymerase pol-γ. NRTI phosphorylation seems to correlate with mitochondrial toxicity, but experimental evidence is lacking. Transgenic mice (TGs) with cardiac overexpression of thymidine kinase isoforms (mitochondrial TK2 and cytoplasmic TK1) were used to study NRTI mitochondrial toxicity. Echocardiography and nuclear magnetic resonance imaging defined cardiac performance and structure. TK gene copy and enzyme activity, mitochondrial (mt) DNA and polypeptide abundance, succinate dehydrogenase and cytochrome oxidase histochemistry, and electron microscopy correlated with transgenesis, mitochondrial structure, and biogenesis. Antiretroviral combinations simulated therapy. Untreated hTK1 or TK2 TGs exhibited normal left ventricle mass. In TK2 TGs, cardiac TK2 gene copy doubled, activity increased 300-fold, and mtDNA abundance doubled. Abundance of the 17-kd subunit of complex I, succinate dehydrogenase histochemical activity, and cristae density increased. NRTIs increased left ventricle mass 20% in TK2 TGs. TK activity increased 3 logs in hTK1 TGs, but no cardiac phenotype resulted. NRTIs abrogated functional effects of transgenically increased TK2 activity but had no effect on TK2 mtDNA abundance. Thus, NRTI mitochondrial phosphorylation by TK2 is integral to clinical NRTI mitochondrial toxicity.** ***(Am J Pathol 2007, 170:865–874; DOI: 10.2353/ajpath.2007.060655)***

Mitochondrial toxicity of nucleoside reverse transcriptase inhibitors (NRTIs) limits treatment of human immunodeficiency virus (HIV) infection.[1,2] Alternative therapeutic choices, including NRTI-sparing regimens, obviate some toxic manifestations[3] but are limited in other ways or are less effective virologically. The global hypothesis for this project is that the initial phosphorylation of NRTIs is critical to both their pharmacological function and mitochondrial toxicity. With respect to their phosphorylation in mitochondria, pathways used by NRTIs are identical to those used by native nucleotides.[4] In humans, mitochondria are capable of importing unphosphorylated pyrimidine deoxyribonucleosides (and NRTIs) directly via the equilibrative nucleoside transporter (ENT1).[5–9] Three sequential phosphorylations follow intramitochondrially to generate the active deoxyribonucleoside- or NRTI triphosphate. The critical first intramitochondrial phosphorylation of pyrimidines is performed by the mitochondrial isoform thymidine kinase 2 (TK2, a nuclear-en-

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coded, mitochondrially localized TK in mitotically quiescent cells). The cytoplasmic isoform, TK1 (EC 2.7.1.21), phosphorylates deoxythymidine (dThd) and deoxyuridine (dUrd) in the cytoplasm of cells. TK1 is richest in cells that undergo rapid turnover, such as epithelia, and is substantially less abundant in mitotically quiescent cells, such as cardiac myocytes. Mutations in genes of human TK2 are linked to inherited mitochondrial (mt) DNA depletion syndromes (MDS)[10,11] (MIM 251880), a heterogeneous group of inherited disorders sharing a common reduction in mtDNA copy number and diverse pathological features.

In some circumstances, cytosolically phosphorylated nucleotide diphosphates (dNDPs) are capable of being imported intramitochondrially by the deoxynucleotide carrier (DNC).[12] dNDPs are then triphosphorylated intramitochondrially to active NRTI triphosphates and bypass mono- and diphosphorylation intramitochondrially. Recently, we investigated this pathway of dNDP entry with respect to NRTI mitochondrial transport *in vivo* by using cardiac overexpression of DNC in transgenic mice (TGs). DNC TGs exhibited mitochondrial dysfunction and structural abnormalities that were worsened by pyrimidine NRTI administration.[13,14]

Not all NRTIs cause mtDNA depletion, and toxicity of certain NRTIs seems to be tissue-specific. Mechanism(s) involved in NRTI mitochondrial toxicity have yet to be fully elucidated. Generation of TGs that overexpress TK2 in the murine heart was accomplished using the $\alpha$-MyHC promoter and methods established by Robbins and colleagues,[15] which we applied to our system previously[14,16–18] and here. Cardiac targeting of human (h)TK1 transgenesis was used as a control in this study. Although its TK function is fairly similar, TK1 is not expressed in myocardial cells. Experiments addressed the specific role of TK2 in cardiac mitochondrial processing of nucleotides and NRTIs. The resultant TK2 TG exhibited robust, targeted expression in the heart. Transgenic TK2 increased mtDNA replication in the heart and contributed to functional defects that occurred with NRTIs. These *in vivo* experiments addressed the hypothesis that TK2 pathophysiology impacts toxicity of NRTIs in the heart at the pharmacological level of their monophosphorylation.

## Materials and Methods

### Generation of $\alpha$-MyHC/TK1 and TK2 TGs

Established methods were used essentially as described previously[13,14,16,18] and applied to the TK1 and TK2 cDNA constructs from Wang and Eriksson.[19–22] The resulting constructs contained the N-terminal mt signal sequence (in case of murine TK2[22]) and the intact N- and C-terminal cDNA sequences (in the case of human TK1).[20,21]

### Treatment Protocols

Procedures complied with Emory Institutional Animal Care and Use Committee and National Institutes of Health guidelines. Antiretroviral drugs were from the manufacturers or from the Emory Center for AIDS Research Pharmacology Core (under the direction of Dr. Raymond Schinazi, Veteran's Administration Medical Center, Decatur, GA). Dosing was done by daily gavage (morning) at doses that resemble human therapy. Highly active antiretroviral treatment (HAART) doses included 3′-azido-2′,3′-dideoxythymidine (AZT) = 0.22 mg/day; 3TC = 0.11 mg/day; and indinavir = 0.9 mg/day or vehicle controls. Morbidity and mortality from the procedure were negligible. At day 35, measurements were made, animals were sacrificed, and samples were retrieved and stored for analyses later.

### Genotyping

For the respective TG lines $\alpha$-MyHC/TK1 and TK2, the presence of the transgene was detected in the founders and their offspring using Southern blotting and real-time polymerase chain reaction (PCR), essentially, as we have done in the past.[14,16,18]

### Mortality of TK TG Lines

Based on survival postpartum, mortality statistics were calculated according to standard methods on TG lines and on their wild-type (WT) littermates.

### Assays for TK Activities in TK1 and TK2 TG and WT Hearts

Activity of TK was determined in total protein extracts of frozen heart samples (containing both the cytosolic and mitochondrial soluble proteins) using an established radiochemical method previously described.[23] In brief, 0.2 to 200 $\mu$g of total cardiac protein extract was incubated in buffer containing 50 mmol/L Tris-HCl, pH 7.6, 5 mmol/L $MgCl_2$, 5 mmol/L ATP, 10 mmol/L NaF, 2 mmol/L dithiothreitol, 0.5 mg/ml bovine serum albumin, and 40 $\mu$mol/L [$^3$H]Thd. Aliquots from the reaction mixtures were spotted onto Whatman DE-81 filters (Fisher Scientific GTF, Västra Frölunda, Sweden) at three time points, and filters were washed in 5 mmol/L ammonium formate. Quantification of the radiolabeled product was accomplished by liquid scintillation counting. Results were expressed in pmol of monophosphates formed per mg of tissue per 30 minutes. Activity measurements were repeated at least twice, and results are presented as mean ± SE.

### TG Gene Copy Analysis

Four transgenic lines each were established for the targeted overexpression of hTK1 and TK2, respectively. To determine the relative copy number in each line, the level of hTK1 or TK2 was analyzed semiquantitatively from murine tail DNA extracts using real-time PCR and Light Cycler TaqMan master kit (Roche Diagnostics Corp., Indianapolis, IN). Target genes were amplified using specific primers for hTK1 (forward: 5′-CAAATCTCCCGC-

CAGTCA-3′ and reverse: 5′-CCGACCGCTTTAAACCAC-3′, Universal ProbeLibrary probe no. 70; Roche Diagnostics Corp.), TK2 (forward: 5′-TACGAGGAGTG-GCTGGTCA-3′ and reverse: 5′-GTTGTGGTCAGCCT-CAATCA-3′, Universal Probe Library probe no. 33; Roche Diagnostics Corp.), and housekeeping gene, GAPDH (forward: 5′-GATGCTACAAGCAGGCCTTT-3′ and reverse: 5′-GCAGAAAGCAAGGGCAAA-3′, Universal ProbeLibrary probe no. 4; Roche Diagnostics Corp.). DNA amplification was performed using LightCycler 480 (Roche Diagnostics Corp.) on individual tissues extracted from at least four mice within each line. Relative copy number dosage was normalized to TK2 (single copy gene) from WT.

### *mtDNA and Nuclear DNA (nDNA) Quantification in Heart Tissue Using Real-Time PCR*

Methods used were based on modifications of those used by others.[24,25] Snap-frozen heart tissue (10 to 20 mg) was thawed and cut into small pieces, and total DNA was extracted using a MagNA Pure system and reagents (Roche Diagnostics Corp.) following the manufacturer's protocol. DNA concentration was determined by using a microtiter plate reader/spectrophotometer (Molecular Devices, Carlsbad, CA), and DNA was diluted in $ddH_2O$ for mtDNA and nDNA amplification.

DNA sequences for primers and probes used for quantization of mitochondrial and nuclear DNA with real-time PCR technique were adapted from others.[25] The accessory subunit of murine mitochondrial DNA polymerase γ (*ASPG*) and mouse mitochondrial cytochrome oxidase subunit 1 (*COX*) were the subjects of separate quantization of mouse nuclear and mitochondrial DNA, respectively. The mitochondrial forward primer (5′-TCGT-TGATTATTCTCAACCAATCA-3′) and reverse primer (5′-GCCTCCAATTATTATT GGTATTACTATGA-3′) were used to amplify the target segment of *COXI* gene. Hybridization probes for this gene were 3′-fluorescein (5′-AACCAGGT-GCACTTTTAGGAGATGACCF-3′) and 5′-LC Red 640 3′-phosphate-blocked (5′-L-AATTTACAATGTTATCGTA-ACTG CCCATGCP3-′). The nuclear forward primer (5′-GGAGGAGGCACTTTC TCAGC-3′) and reverse primer (5′-GAAGACCTGCTCCCTGAACAC-3′) were used to amplify the nuclear *ASPG* gene. A 3′-fluorescein labeled oligonucleotide (5′-GCGCTTTGGACCTTTGGGTGTAG-F3′) and a 5′-LC Red 640 3′-phosphate-blocked (5′L-G-TTACGAAAGAACCTAGCCTCACAGTGGT-P3′) oligonucleotide were used as hybridization probes for the nuclear gene.

Real-time PCR was performed in duplicate for each amplicon following methods used by others[24,25] with some modifications. Briefly, each reaction contained 4.0 μl of master mix (LightCycler 480 Genotyping Master; Roche Applied Science, Indianapolis, IN), 1.0 μmol/L of each primer, 0.2 μmol/L of each probe, 5 μl of $ddH_2O$, and 5 μl of diluted DNA template.

Amplification was performed in a LightCycler 480 (Roche) and consisted of a denaturation step (8 minutes), 50 cycles of amplification (95°C for 8 seconds, 55°C for 10 seconds followed by single fluorescence acquisition, and 72°C for 10 seconds), a melting curve (95°C for 30 seconds with a ramping rate of 4.8°C/second, 45°C for 61 seconds with a ramping rate of 2.5°C/second and 95°C for 0 seconds), and a cooling cycle (40°C). Temperature ramping rates were 2.5°C/second unless noted.

Standard DNA curves for quantization of the LC products were used. Both mitochondrial and nuclear target sequences were PCR amplified and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Each plasmid was linearized by digestion with *Hind*III restriction endonuclease (Roche) following the manufacturer's protocol. Each fragment insert was then PCR amplified using their corresponding original primers and ran out on 2% agarose gels to ensure quality and proper size of the inserts.

Serial dilutions were made from products and the copy number was calculated based on the molecular weight of plasmid plus the insert, and PCR reactions were performed to construct the standard curve for mitochondrial and nuclear DNA. These standard curves were saved as external standard curves and were later used to quantify the mtDNA and nuclear DNA after each run. Samples were run in duplicate. PCR products of mtDNA and nDNA were quantified by using the corresponding external standard.

### *Immunoblotting of Mitochondrial Complex I*

Methods used resembled those used by us recently[26] and applied to isolated cardiac mitochondria obtained from TG and WT hearts.[14] In brief, mitochondria were isolated using the Mito Iso kit (Sigma, St. Louis, MO), and protein concentrations were determined with bicinchoninic acid assay. Protein (50 μg) was loaded into each well of a 12% Tris-HCl Ready Gel (Bio-Rad, Hercules, CA) and subjected to electrophoresis (1.5 hours, 80 V). Transfer to Bio-Rad Immuno-Blot polyvinylidene difluoride membrane (0.2 μm) was performed for 2 hours at 80 V. The membrane was blocked overnight at 4°C and washed six times with a 1× Tris-buffered saline-Tween 20 (each 5 minutes). The membrane was probed simultaneously with antibody to the 17-kd subunit of complex I (Anti-Ox Phos complex I, 17-kd, 0.5 μg/ml; Molecular Probes, Portland, OR) and anti-porin antibody (1 μg/ml; Molecular Probes) for 1 hour at room temperature (each diluted in 2% blocking buffer). The membrane was washed four times with 1× Tris-buffered saline-Tween and probed (1 hour) at room temperature with the secondary antibody (goat anti-mouse IgG-horseradish peroxidase; Santa Cruz Biotechnology, Santa Cruz, CA), followed with six washes with 1× Tris-buffered saline-Tween. Luminescent reagent (Luminol; Santa Cruz Biotechnology) was applied to the membrane (1 minute). The membrane was developed on chemiluminescent film (Hyperfilm ECL; Amersham, Arlington Heights, IL). Densitometry of the bands was quantitated with GelExpert 3.5 software (Nucleo Tech, San Mateo, CA). The 17-kd subunit of complex I was normalized to that of porin.

### *Fine Structure Pathological Evaluations with Transmission Electron Microscopy (EM) Analysis of Mitochondrial Damage of TK TG Lines*

Ultrastructure ($n = 12$) was evaluated using transmission EM to support data from echocardiography and magnetic resonance imaging (MRI) using methods that resemble those used regularly in the laboratory.[27] Sections (0.5 $\mu$m) were cut with glass knives and stained with toluidine blue for orientation. Ultrathin (900 Å) sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and viewed on a Philips EM201 microscope (Philips Medical Systems, N.A., Bothell, WA) with evaluation and photography by blinded observers. Representative high-power views were included to demonstrate ultrastructural features. Each EM photograph was reviewed independently by two investigators for the presence of structurally abnormal mitochondria, increased numbers of mitochondrial profiles per field, intramitochondrial lamellar bodies, abnormal cristae density, cristae reduplication, mitochondrial swelling, and intramitochondrial paracrystals as performed by others.[28]

### *Enzyme Histochemistry for Succinate Dehydrogenase (SDH) and COX in TG and WT Mice*

SDH and COX histochemical analyses are reliable for visualization of normal mitochondria and for identification of disrupted mitochondrial biogenesis. SDH is a substrate-specific mitochondrial oxidative enzyme that reflects the utilization of a metabolic intermediary in the Kreb's cycle. The reduction of a tetrazolium salt to an insoluble, colored (dark blue) formazan product correlates to level of type I skeletal muscle fibers, or actively proliferating mitochondria associated with most genetic and acquired mtDNA defects. COX in tissue sections causes the oxidation of 3,3′-diaminobenzidine to form a brown precipitate at the site of the enzyme in mitochondria. Specific methods for histochemistry are from standard protocol used at Emory University with minor modifications.[29]

### *Echocardiography in TK TG Lines and WT*

For echocardiography observations in the protocols with hTK1 and TK2 TG lines, left ventricle (LV) mass quantization was performed and normalized using echocardiography in age- and gender-matched (littermate) WT and TGs as we have done in the past.[27]

### *Cardiac MRI in TK2 TGs and WT Littermates with NRTI Treatment*

To determine LV cavity volume, TK2 TGs ($n = 3$; two treated, one untreated) and WT littermates ($n = 3$; two treated, one untreated) were evaluated via MRI at 16 to 20 weeks using methods that resembled those used by us in the past,[13,16] including a 4.7T Varian/INOVA (200/33) spectroscopy and imaging system (Varian, Palo Alto, CA).

**Table 1.** TK Gene Copy Dosage

| TK | Line | Dosage* | Copy number |
|---|---|---|---|
| hTK1 | A line | 3.0 | Medium |
| hTK1 | C line | 3.9 | High |
| hTK1 | D line | 2.0 | Low |
| hTK1 | E line | 5.3 | High |
| Native TK1 | WT | 1.0 | Not expressed |
| TK2 | A line | 3.9 | High |
| TK2 | B line | 1.7 | Low |
| TK2 | C line | 2.4 | Medium |
| TK2 | D line | 1.8 | Low |
| TK2 | WT | 1.0 | Single copy |

*Relative copy number normalized to TK2 WT (single copy gene).

### *Statistical Analysis*

For mtDNA quantification, data were expressed as the ratio of mean value of the mtDNA measurement to the mean value of nDNA divided by 1000, and the resultant values are expressed as mean ± SE. A value of $P < 0.05$ was considered significant. Echocardiographic determinations from all groups were compared by analysis of variance.[27]

## *Results*

### *General*

Targeted transgenesis of hTK1 and TK2 was accomplished in four lines each. Animals bred true for six generations before experimental use. No gross phenotype was recognized in TGs or littermates. No changes in behavior, growth, maturation, breeding behavior, or Mendelian distribution of TG were found in any of the lines. Table 1 documents the relative increase in TG lines in both hTK1 and TK2 TGs. In general, transgenesis revealed robust expression of the TG with more than a fivefold increase for TK1 maximally and up to a fourfold increase in TK2 maximal gene copy. In reporting the gene copy for TK1, assessment is based on human TK1 gene sequence, which is slightly different from mouse TK1 and was not detectable in WT, although TK1 is a single copy endogenous gene (Table 1). Longevity was similar in both WT and TGs and resembled FVB/n longevity (not shown).

### *Assays for TK Activities in hTK1 and TK2 TGs*

Activity of the transgenically expressed enzymes was *prima facie* evidence for successfully targeted transgenesis and underpinned authenticity of the phenotype. Compared with activity from whole tissue samples from WT littermates, TK enzyme activity was dramatically higher in heart samples from TGs (Table 2). TK enzyme activity in TK1 TGs increased 3 logs more than WT. Because TK activity in WT heart is minimal,[4] increased

**Table 2.** TK Activity in Myocardial Extracts from WT and TG

| Mice | TK activity (pmol/minute/mg) | Fold changes |
|---|---|---|
| WT ($n$ = 4) | 6.6 ± 1.2 | 1 |
| TK2 TG B line ($n$ = 4) | 1994 ± 430 | 302 |
| TK1 TG C line ($n$ = 3) | 30,202 ± 8554 | 4576 |

Assays were performed with $^{3}$H-labeled thymidine as substrate using total heart tissue extracts as enzyme source as described in Materials and Methods. The values were mean ± SE; $n$ indicates the number of mice.

activity seen in hearts of hTK1 TGs documented effective TK1 transgenesis.

Because of the large number of TGs and drug combinations, we selected medium expressors and a commonly used NRTI combination for use in studies to determine mitochondrial biogenesis and cardiac function. TK enzyme activity in heart samples from TK2 TGs was 300-fold that of WT. Notably, quadriceps femoris samples from either TK1 or TK2 TGs showed TK activity that was unchanged from WT controls, supporting the data that indicated transgenesis was authentically cardiac-targeted (data not shown).

## *mtDNA/nDNA Abundance*

mtDNA depletion is considered a hallmark of clinical NRTI toxicity. Its measurement in blood cells was used as a surrogate to diagnose mitochondrial toxicity in tissues.[1,24] Steady-state abundance of mtDNA (relative to nDNA) was determined directly in cardiac samples from TGs and WTs undergoing HAART treatment ($n \geq 7$ per cohort). hTK1 TGs treated with combined antiretroviral drugs (that included AZT) showed no effect on mtDNA/nDNA ratio (Figure 1A). All mtDNA/nDNA ratios for TK1 and TK2 studies were normalized for comparability of studies.

As mentioned, the TG with moderately increased TK2 gene copy was selected for treatment protocols (1.7-fold increase; B-line; see Table 1) and treated with HAART. That TG exhibited ~40% higher mtDNA/nDNA ratio than that mtDNA ratio from hearts of WTs (treated or untreated) ($P < 0.001$) (Figure 1B). Untreated TK2 TGs also showed ~30% higher mtDNA/nDNA compared with that of HAART-treated or untreated WT ($P < 0.001$). However, no change was detected in the mtDNA/nDNA ratios in TK2 TGs with or without treatment.

## *Mitochondrial 17-kd Subunit of Complex I Immunoblot Quantization*

Abundance of polypeptides encoded by mtDNA (specifically those elements of complex I of the electron transport chain) was determined by immunoblot analysis of cardiac mitochondria isolated from TK2 TG and WT hearts with and without NRTI treatment. Detection of the 17-kd subunit of complex I from mitochondrial samples was normalized to porin signals and then converted to arbitrary units for comparisons of multiple blots (Figure 2).

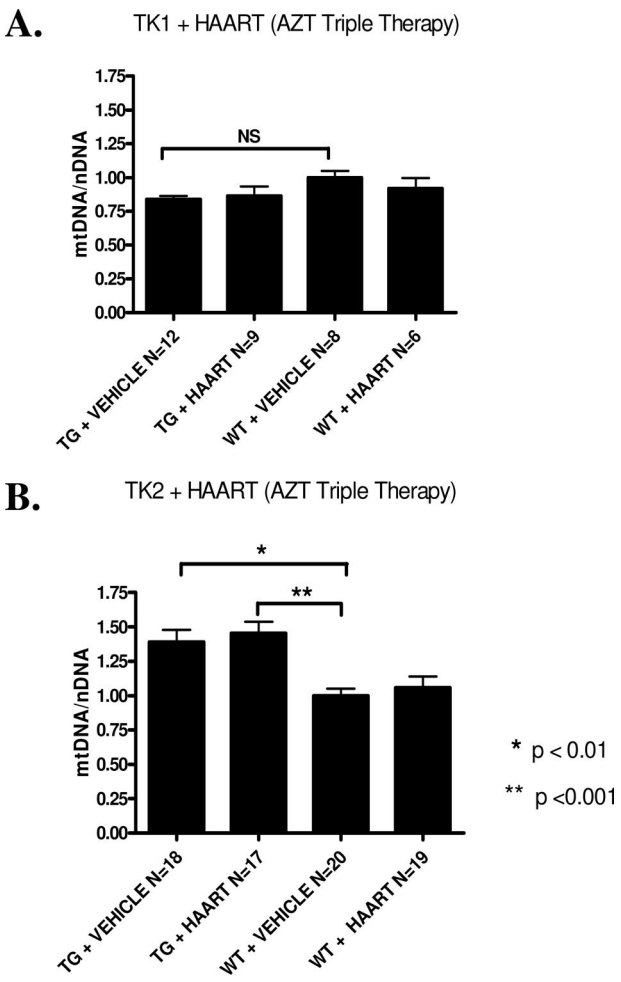


**Figure 1.** Real-time PCR mtDNA/nDNA ratios with NRTI treatment: TG and WT cohorts were treated with HAART [AZT, 3TC, and indinavir (IDV) for 35 days] or with vehicle control for 35 days. Tissue samples were analyzed using real-time PCR. **A:** TK1 + HAART resulted in no significant change from WT. **B:** An increase in mtDNA/nDNA ratio was found with TK2-HAART compared with WT.

Using this approach, AZT-based combination therapy caused no detectable change in steady-state abundance of the 17-kd subunit of complex I in mitochondria from heart samples of TK1 TGs (Figure 2A). In comparison, mitochondria isolated from TK2 TG hearts (untreated) exhibited a small (~11%) but significant increase in the 17-kd subunit of complex I compared with that found in WT cardiac mitochondria (Figure 2B). With the addition of NRTI therapy, the 17-kd subunit of complex I steady-state abundance in TK2 TGs decreased and reverted back to levels found in WTs.

## *Ultrastructural Features of Mitochondria in TG Hearts*

EM is an established, semiquantitative method for analysis of NRTI toxicity[14,27] and for determination of mitochondrial volume.[30] EM was used by other investigators to analyze mouse pup cardiomyocytes after NRTI treatment of the dam.[31] EM data here supported biochemical and molecular findings (above) and echocardiography

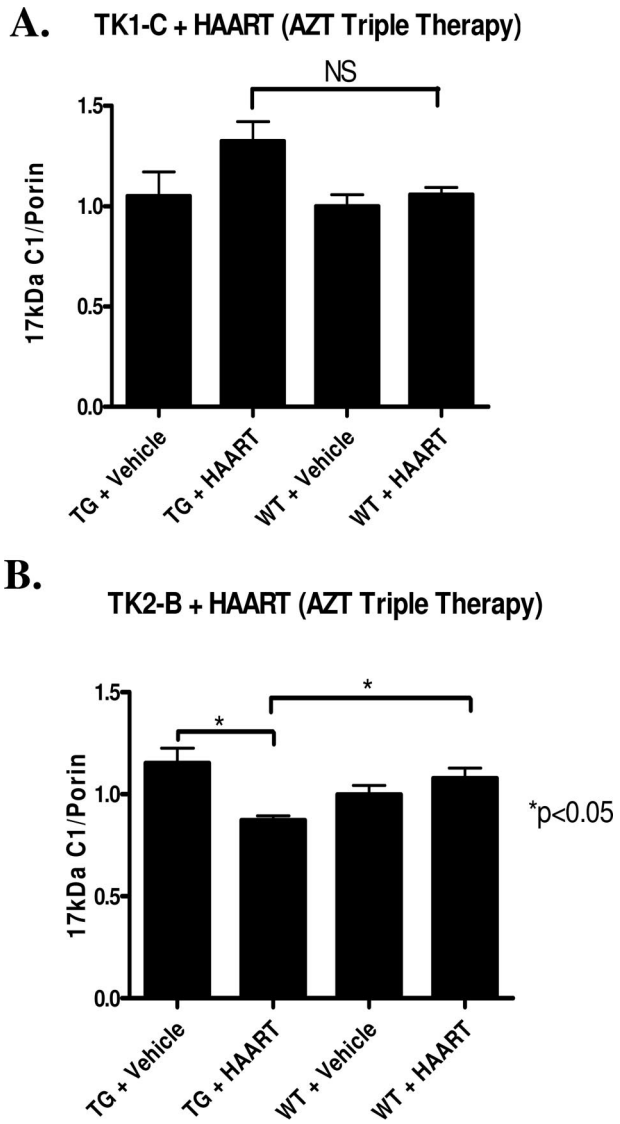


**Figure 2.** Steady-state abundance of 17-kd subunit of complex I polypeptide by Western blot in TK1 (**A**) or TK2 (**B**) TGs with AZT-HAART: densitometric scans were performed using antibody to the 17-kd subunit of complex I and densities normalized to porin control on the same blot. All data are expressed as relative density. A small but significant increase (~11%) in abundance of 17-kd subunit of complex 1 was found in untreated TK2 TGs compared with WT counterparts ($P < 0.01$). Treatment with combined antiretrovirals containing AZT reverted abundance of 17-kd subunit of complex I in TK2 TGs to that found in WT.

and MRI findings (below). hTK1 TGs revealed essentially no ultrastructural changes in the cardiac mitochondria with or without HAART treatments (data not shown). In TK2 TGs, some mitochondria looked spherical or oval-shaped (Figure 3, bottom left), and NRTI treatment seemed to increase mitochondrial density. Interestingly, both density of cristae and size of mitochondria were prominent in TK2 TGs, particularly with HAART (Figure 3, bottom).

## *SDH and COX Enzyme Histochemistry*

Enzyme histochemistry demonstrates the tissue activity of SDH or COX in the heart and skeletal muscle. SDH and

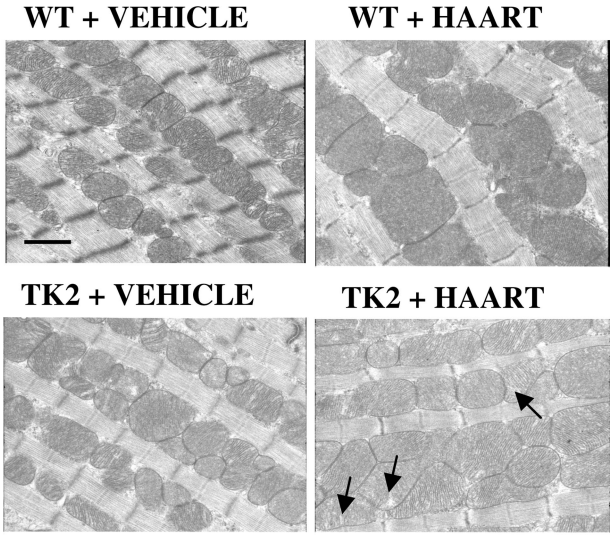


**Figure 3.** Electron photomicrographs of mitochondria from cardiac myocytes of TK2 TG and WT: TK2 overexpression in the heart increased mitochondrial abundance and cristae density (bottom left). The addition of HAART containing AZT resulted in some mitochondrial destruction with some loss of cristae (**arrows**), but abundant densely packed mitochondria remained. Scale bar = 1 $\mu$m. Original magnifications, ×26,000.

COX histochemistry are proven, reliable methods for visualization of normal mitochondria and for evaluation of dysfunctional mitochondrial biogenesis. Frozen cardiac tissue sections from each cohort were stained for SDH or COX enzyme activities using established methods. Heart sections from untreated TK2 TGs exhibited a higher level of SDH activity compared with untreated WTs, as demonstrated by more intense blue staining throughout the TG heart tissue (Figure 4, compare top and bottom left panels, respectively). AZT-HAART treatment of WTs re-

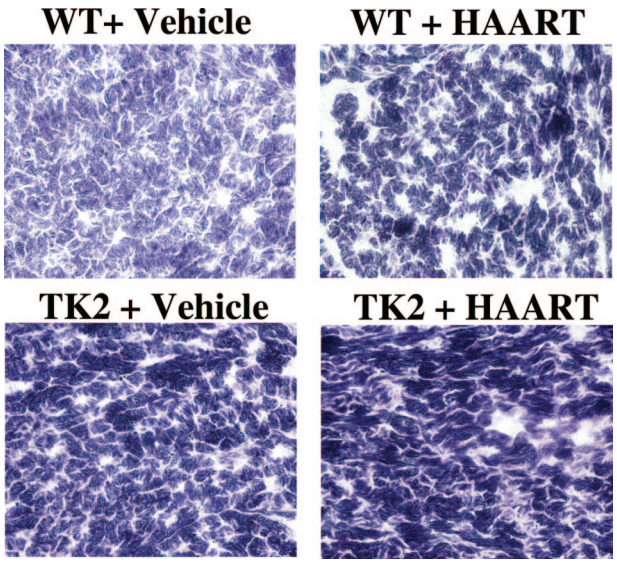


**Figure 4.** SDH histochemical staining in cardiac tissues from TK2 TGs and WTs: frozen cardiac tissues from representative TK2 TGs and WTs, with or without AZT-HAART (35 days), were sectioned and stained for SDH enzyme activity. Representative stained tissues for untreated WT (top left) or TK2 TGs (bottom left) and AZT-HAART-treated WT (top right) or TK2 TGs (bottom right) are shown. Increased dark blue staining is indicative of increased SDH enzyme activity. Darkest staining was found in TK2 + HAART sample (bottom right).

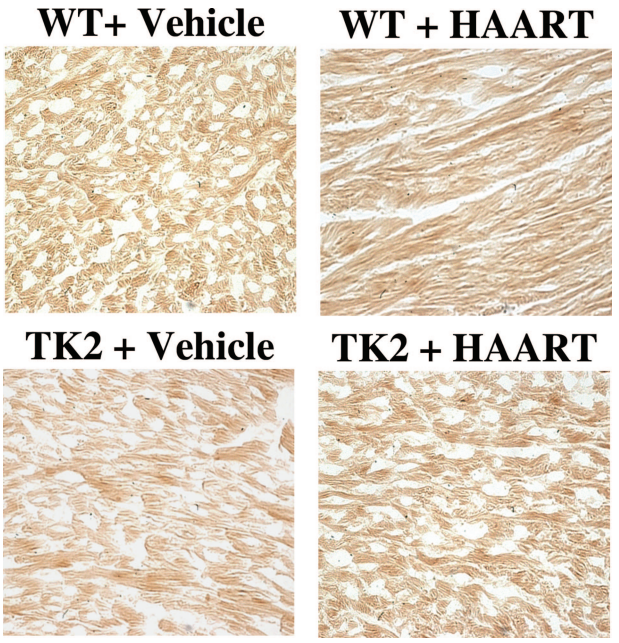


**Figure 5.** COX histochemical staining in cardiac tissues from TK2 TGs and WTs: frozen cardiac tissues from TK2 TGs and WTs, with or without AZT-HAART (35 days), were sectioned and stained for SDH enzyme activity. Representative stained tissues for untreated WT (top left) or TK2 TGs (bottom left) and AZT-HAART-treated WT (top right) or TK2 TGs (bottom right) are shown. Sites of COX enzyme activity have dark brown to red precipitation.

sulted in a clear increase in SDH activity compared with that seen in cardiac sections from untreated WT controls (Figure 4; compare top left and right panels, respectively). AZT-HAART treatment of TK2 TGs resulted in the most intense SDH staining (Figure 4, bottom right). In contrast, the COX histochemical staining of frozen cardiac tissue samples from the same cohorts demonstrated minimal changes in COX (Figure 5).

### *Echocardiographic Evaluation of LV Mass in Hearts of TK1 and TK2 TGs with and without Antiretroviral Therapy*

Both LV mass and left ventricle end diastolic volume (LVEDV) reflect cardiac function. An increase in either parameter is characteristic of cardiac dysfunction resulting in increased LV mass and dilated LV cavity. Echocardiographic data were obtained in both TK1 and TK2 TG lines with and without antiretroviral combinations (Figure 6). TK1 TGs exhibited no echocardiographic phenotype (normalized LV mass; mg/g body weight) with or without the AZT-based combination when compared with LV mass found in control cohorts (Figure 6A). In contrast, echocardiographic studies with TK2 TGs undergoing a similar antiretroviral protocol revealed increased LV mass after 35 days of treatment. NRTI-treated TK2 TGs exhibited a 24% increase in LV mass compared with that of untreated TK2 TGs. In addition, coincidence of treatment and TG resulted in ~50% increased LV mass greater than that of untreated WTs (Figure 6B).

### *Cardiac MRI of TK2 TGs and WT Littermates with and without NRTI Treatment*

LV dilation was defined quantitatively by MRI in representative mice. MRI images of hearts of TK2 TGs revealed increased LVEDV compared with LVEDV found in WT (Figure 7). In untreated TK2 TGs, LVEDV increased 41% more than levels in WTs. With the addition of combined antiretroviral therapy (TK2 + HAART), LVEDV was 26% more than that of the untreated TK2 TG and 77% more than that in untreated WTs (Figure 7). LVEDV of WT was essentially unchanged by administration of antiretrovirals (Figure 7). Changes in LVEDV related to both TK2- and AZT-based therapy.

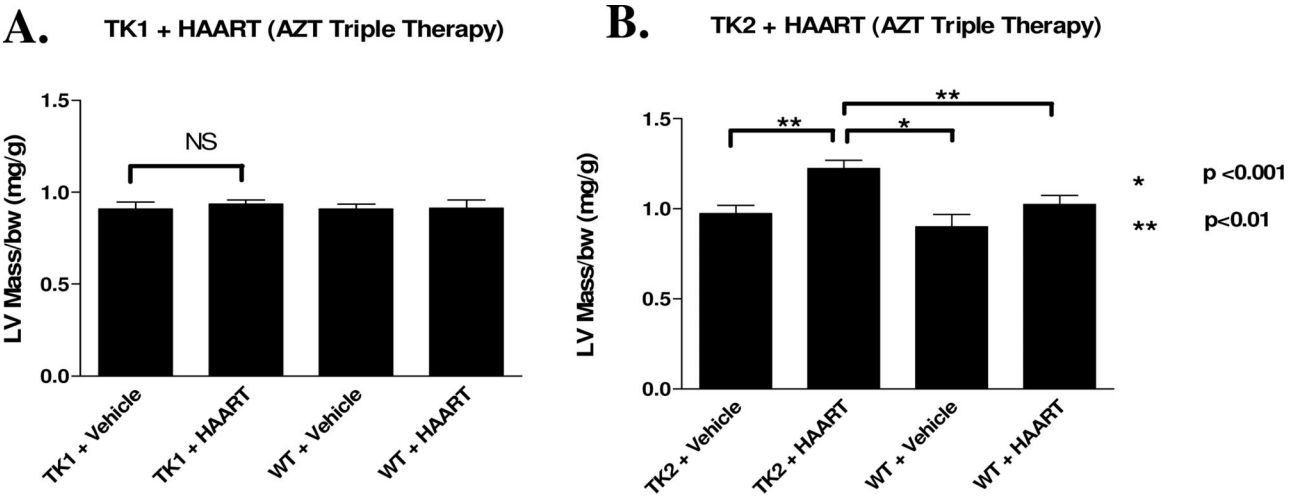


**Figure 6.** Quantitative analysis of echocardiographic images: LV mass was calculated in a blinded manner, code was broken, and data tabulated. **A:** TK1 TGs resulted in no change in LV mass from WT. **B:** TK2 TGs, both untreated and treated, had significant increases (~25 to 50%) in LV mass from WT. Data were normalized to body weight (mg/g) and plotted as mean ± SEM.

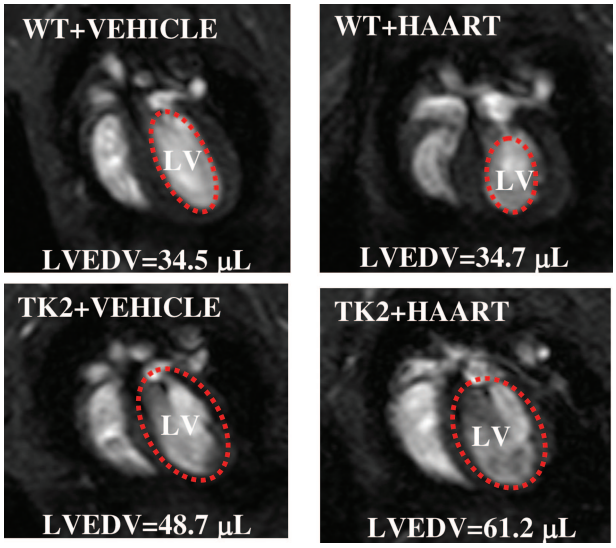


**Figure 7.** Cardiac MRI of TK2 TGs and WT littermates: to evaluate LV cavity volume, TK2 TGs ($n = 3$; two treated, one untreated) and WT littermates ($n = 3$; two treated, one untreated) were evaluated via MRI at 16 to 20 weeks using a 4.7T Varian/INOVA (200/33) spectroscopy and imaging system. MRI images of hearts of TK2 TGs revealed increased end diastolic volume (LVEDV) (48.7 versus 61.2 μl, untreated versus treated, respectively) compared with LVEDV found in WT (~34.5 μl for both treated and untreated). LVEDV of WT was essentially unchanged by administration of antiretroviral therapy.

## *Discussion*

Targeted cardiac TGs are unique biological tools that define features of cardiac dysfunction.[32] A murine transgenic approach helped unravel some features of AIDS heart disease.[27,33,34] Clinically relevant changes were found related to HIV gene products,[16,18] and to antiretroviral therapy in models in which defective mitochondrial biogenesis related to drug toxicity.[35]

In the present study, cytosolic TK1 and mitochondrial TK2 isoforms each were individually overexpressed in the murine heart. mtDNA and mitochondrial polypeptide abundance increased in TGs that overexpressed TK2, but not in those that overexpressed hTK1. Data suggest that activity of TK2, the mitochondrial isoform of TK, influences mtDNA abundance at the level of the nucleotide pool for mtDNA replication. Moreover, mtDNA abundance was disturbed by administration of an AZT-based antiretroviral combination. At first glance, increased mtDNA abundance seems counterintuitive because decreased mtDNA is characteristically reported in mitochondrial dysfunction associated with NRTI toxicity after relatively long-term exposure. The observed increase here may be an attempt by mitochondria to compensate for disrupted mtDNA replication, as is suggested in some mitochondrial genetic disorders. This concept is supported by work from Poirier and colleagues,[36] who eloquently demonstrated *in vitro* that increased mtDNA followed treatment with zidovudine in tissue culture. This finding is described in genetic mitochondrial disease as well.[37] Based on the data presented here, some control of mtDNA replication seems to be exerted at the level of intramitochondrial phosphorylation of Thd and/or deoxycytidine. Steady-state mtDNA abundance and mitochondrial 17-kd subunit of complex I increased with TK2 transgenesis. Experiments here support the critical nature of nucleotide pools and TK2 in mitochondrial biogenesis.

hTK1 had little or no effect on any mitochondrial parameters examined here, yet it served as a critical control. Transgenically induced high expression of cytosolic TK1 had no molecular, biochemical, pathological, or physiological effect on the heart. On this basis, TK1 is not involved in NRTI toxicity in the murine model here. Based on high TK activity in extracts of TK1 TG hearts, it is reasonable to deduce that formation of AZT-MP in cardiac cytosol does not interfere with mtDNA synthesis. It also follows reasonably that TK2 may regulate mtDNA precursor pools and TK2 enzyme activity may relate to deleterious effects of NRTIs in myocardium of humans.[35] Conversely, mtDNA precursor pools (and TK2 activity in the heart) may impact mitochondrial aspects of cardiac dysfunction in forms of heart disease unrelated to HIV/AIDS[38] and thus may merit exploration.

The biological effect of any transgenic manipulation may be downstream of its transgenic function, particularly if the transgenic polypeptide product is an enzyme involved in generating biological precursors for a series of reactions. In the case of TK2, its monophosphorylated product (dTMP) is a precursor of dTTP for mtDNA replication. Moreover, it has been shown previously that NRTI-MPs inhibit DNA pol-γ, albeit not competitively.[39] AZT-TP is a relatively poor substrate for mtDNA pol-γ.[40] This may underscore the importance of precursor depletion in biological processes that ultimately lead to mtDNA changes.[42] Based on increased mtDNA abundance found in TK2 TGs (see above) and previous data that showed AZT depletion of mtDNA *in vivo*,[42,43] it may be reasonable to expect an interplay between transgenically increased mtDNA and pharmacologically induced mtDNA depletion and resultant effect on mitochondrially encoded polypeptides.

Direct measurement of nucleotide or NRTI TP pools is daunting *in vivo*.[44] To address the question, we chose an alternatively informative approach that defined TK enzyme activity and abundance of mtDNA. As suggested by Bianchi and colleagues[45] and Song et al,[46] the intramitochondrial dThd pool also is regulated tightly by TK2 in the system used here. Conversely, cytoplasmic monophosphorylation of dThd by TK1 does not impact intramitochondrial dThd pools in the TK1 TG model. In the future, an engineered gene of TK1 that contains a mitochondrial targeting sequence may serve as an interesting follow-up experiment to elucidate this point further.

Based on the work of colleagues,[12] we recently addressed NRTI toxicity at the level of a mitochondrial transport of nucleotides (DNC).[13,14] We developed and used TGs with cardiac-targeted DNC and treated them with various antiretroviral combinations that included pyrimidine nucleoside analogs AZT, 3TC, or d4T. Those studies revealed that cardiac dysfunction resulted from AZT and d4T (alone or in combined therapy) but not from 3TC monotherapy nor from an NRTI-sparing combination that contained no nucleoside analogs. Data implicated the chemical structure of the pyrimidine NRTI triphosphate

and its interaction with the active point of DNA pol-γ as a toxic focus with d4T and AZT.

The DNA pol-γ hypothesis[2] has been developed and refined throughout the years[1,47] to help define events in acquired mtDNA depletion caused by antiretroviral nucleosides for AIDS.[28] Pharmacological and cellular events that relate to abundance of phosphorylated NRTIs and normal mtDNA precursors in the intramitochondrial compartment were considered important initially but now are becoming crucial biological factors in understanding the downstream events in inhibition of DNA pol-γ and in resultant mtDNA depletion.[41,48,49] Accordingly, the first intramitochondrial pyrimidine phosphorylation (by TK2) is a parsimonious upstream site to influence homeostasis of mtDNA replication.[42]

Genetic mtDNA depletion syndromes support the models here and *vice versa*. MDS[50,51] yield a common phenotype that resembles NRTI toxicity *in vivo*. In mtDNA depletion from MDS, mitochondrial and cytoplasmic nucleotide pools are disturbed at the level of kinases, polymerases, and other proteins. Studies here served as proof of principle because similar phenotypes were generated experimentally using targeted transgenesis and NRTI administration to create similar nucleotide pool imbalances.

In summary, this study used TGs that individually expressed mitochondrial (TK2) or cytoplasmic (hTK1) isoforms of TK in the murine heart to define *in vivo* events controlling mtDNA replication and NRTI toxicity. In TK2 TGs, mtDNA abundance, mitochondrially encoded polypeptide abundance, and SDH enzyme activity increased in parallel with increased TK2 enzyme activity and transgene copy number. Increased mtDNA abundance with TK2 overexpression indicated that phosphorylation of nucleotides used in mtDNA synthesis played a *de facto* role in cardiac mtDNA homeostasis *in vivo*. TK1 transgenesis, even at very high levels, had no effect. NRTI administration abrogated some functional effects of transgenically increased TK2 activity, but NRTIs had no effect on TK2 TG mtDNA abundance. Taken together, pyrimidine substrate availability for TK2 and pyrimidine triphosphate availability for DNA pol-γ remain crucial in both mtDNA biogenesis and in the pathogenesis of mitochondrial toxicity from NRTIs.

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